

The University of Chicago

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LACTOBACILLI OF HUMAN
ORAL ORIGIN

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

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ANTIGENIC COMPONENTS OF LACTOBACILLI OF HUMAN ORAL ORIGIN

NED B. WILLIAMS

From the Walter G. Zoller Memorial Dental Clinics and Department of Bacteriology and Parasitology, University of Chicago, Chicago, Illinois

The heterogeneity of the oral lactobacilli has been verified by many investigators, but progress in efforts to find some order within the group has been slow because of the difficulty in attaining methods for consistent identification of the kinds or types most frequently associated with the early carious lesions in teeth. The methods most frequently used up to the present for separation of the various oral strains have been based on cultural, morphological, and fermentative characteristics (Hadley and Bunting, 1930; Hadley, 1933). A report by Hadley and Bunting (1932) described the results of a study designed to determine the most frequently occurring groups in cultures from tooth scrapings and saliva samples from persons with one or more carious lesions. They found that their Types I and III were present in more of the cultures than the other two morphological types described by them. A similar type of study conducted by Bradel and Blayney (1940) with material from the surfaces of teeth, specifically from dental plaques at loci corresponding to the most frequent sites of the early caries of enamel, revealed a predominance of organisms which were identified morphologically and culturally as Types I and II of Hadley and Bunting (1930). The occurrence of a variety of forms intermediate between the various de-

scribed morphological and colonial types, as well as considerable variability in the fermentation reactions of freshly isolated cultures, renders these criteria inadequate for clear separation and identification of groups, types or varieties.

Although the above criteria are insufficient, the application of other possible methods such as immunological techniques has been limited. Most workers who have applied serological methods have done so in making comparisons between oral and intestinal forms. Hilgers (1924) found that oral and intestinal strains could not be distinguished by the complement-fixation test. McIntosh and associates (1924), as well as others (Hadley and Bunting, 1930; Hilgers, 1924; Sierakowski and Zadjel, 1924; Rosebury, Linton and Buchbinder, 1929; Warner and Arnold, 1941) were unsuccessful in their efforts to separate cultures of lactobacilli from these two sources by agglutination and agglutinin absorption tests. However, Morishita (1929) and Anderson and Rettger (1937) interpreted the results of their own agglutination and agglutinin absorption tests to indicate that the oral and intestinal strains are separable serologically. Harrison and Opal (1944) contributed evidence of homogeneity between the oral and intestinal lactobacilli and, in a study of eight paired oral and intestinal strains isolated from children, found that the oral and intestinal strains were indistinguishable morphologically and were identical in precipitation and biochemical reactions.

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The author is not aware of any use of the complement-fixation test for separation of the oral forms, but agglutination and agglutinin absorption, and recently precipitation tests, have been utilized. Morishita (1929) and Howitt (1930) reported that they were able, by agglutination and agglutinin absorption tests, to establish groupings among their oral cultures, but no details of these groupings were described. The first demonstration that the precipitation test could be employed in differentiation of oral forms was reported by Harrison, Zidek and Hemmens (1939). Type-specificity was shown by using bacterial carbohydrate extracts in test tube reactions with anti-serums from rabbits immunized with whole cell vaccines. Strains of oral lactobacilli were classified by them into two groups: one containing four or more immunological types, the other comprising immunologically heterogeneous strains.

The evidence, from carbohydrate precipitation tests, that immunologically distinct groups exist among the oral lactobacilli justified a restudy of the agglutination reactions of these organisms. If such relationships could be demonstrated by means of agglutination tests it should be possible to clear up some of the confusion concerning the presence or absence of agglutinative relationships between lactobacilli of oral and intestinal origin. Furthermore, this type of serological method should offer simpler and less time consuming procedures for the extension of studies on the classification of oral lactobacilli. Data from precipitation tests, in which absorbed and unabsorbed serums were used, indicated (Harrison, 1946) that there is some evidence for the existence of overlapping type relationships between many of the oral lactobacilli. This suggests (1) that carbohydrate

extracts may contain more than a single antigen and (2) that such plural antigens might be distributed in a variety of antigenic patterns, such, for example, as those demonstrated among species of the *Salmonella* (White, 1929). Agglutination studies were undertaken to explore these possibilities.

MATERIALS AND METHODS

Cultures.—Detailed study was limited to nine cultures which have been used in previous studies reported from this laboratory.

Data on cultures used.

Lactobacillus culture No.	Isolation date	Hadley (1930) Type
3, 16, 29, 30, 96	July–September 1937	I
4	July 1937	II
9, 19	July–August 1937	III
45	August 1937	II and III

The cultures were originally isolated from the saliva of patients who were being treated for dental caries in the Walter G. Zoller Memorial Dental Clinics, University of Chicago. Because of the previous experience in this laboratory, that lactobacilli undergo changes while being carried routinely in stock culture, broth cultures of each strain were frozen rapidly and stored in the ice-box containing solid carbon dioxide. When vaccines, antigens or carbohydrate extracts were needed from these cultures a frozen stock culture was thawed and an inoculum obtained to begin a broth culture for freezing, the remainder being used to inoculate mediums for the specified purposes. Stock cultures were also maintained by monthly transfer in a modification (Orland, 1946) of the tomato juice-yeast extract broth of Weiss and Rettger (1934) containing 40% tomato juice. When transferred, cultures were incubated at 37 C for three days then stored in the refrigerator.

Antigens.—Cultures were incubated in the tomato juice broth for three to four days at 37 C, centrifuged and washed three times in a sterile 0.85% sodium chloride solution. Suspension was made in sufficient sterile saline to provide a turbidity of 20 on a photometer (Cenco Model No. 12346A, Central Scientific Company), using a 5 cc capacity tubular absorption cell and a blue filter. The suspensions were heated to 60 C for one hour, tested for sterility, and then stored in the refrigerator until used. An adequate quantity of vaccine was prepared at one time to complete the whole course of injections in a given experiment.

The cells for agglutination tests were collected

by centrifugation, washed three times in large volumes of sterile distilled water and resuspended in distilled water to a turbidity reading of 40 to 50 on the photometer. Phenol was added to a final concentration of 0.2% and suspensions were stored in the refrigerator until used. Spontaneous agglutination among the lactobacilli is well known, but occurred only occasionally with the cultures in this study. When a suspension remained partially agglutinated it was filtered through a thick cotton layer under negative pressure and suspensions, thus freed of clumps, usually remained stable. Completely granular suspensions were improved considerably by adding glass beads to the mass in distilled water, then shaking the mixture mechanically for several hours before filtering. All cultures yielded satisfactory suspensions by these means except for one which gave much difficulty because of spontaneous agglutination. None of these methods decreased the usefulness of the organisms as agglutinating antigens and in most instances improved the stability of the bacterial suspensions.

Immunization.—All rabbits were bled prior to the first injection of vaccine and the serums were tested for agglutination of the antigen to be injected. All antisera were prepared in duplicate rabbits.

The schedule of injections consisted of five to ten 0.2 cc amounts of vaccine given intravenously on alternate days. Animals were bled seven to ten days after the final inoculation. If titers of 1:2,560 or 1:5,120 were not obtained, the animals were reinjected, but the total amount of vaccine, used in any one animal, did not exceed three to five cc. Serums, treated with phenol to a final concentration of 0.2% and then stored in the refrigerator, kept indefinitely without formation of precipitate.

Agglutination tests.—All serums were diluted by the doubling dilutions method with 1.7% sodium chloride solution. This modification of the agglutination test procedure was adopted because the antigens had been washed and resuspended in distilled water. When equal parts of serum dilution and antigen suspension were mixed the final concentration of salt was about 0.85%.

A rapid-slide agglutination test was used for preliminary studies of relationships between various cultures before use of the test tube agglutination method. One drop of the bacterial suspension was added to one drop of each of the serum dilutions, from 1:10 through 1:40, in separate hollow ground slides. After mixing the serum dilutions and bacterial suspension with wooden applicators the slides were tilted back and forth at room temperature for about one or two minutes, then reagitated briefly at five minute intervals for a

total period of ten to fifteen minutes. Controls consisted of one drop of the test suspension plus one drop of 1.7% sodium chloride solution. Positive agglutination usually appeared during the first five minutes. Culture suspensions which agglutinated to a lesser degree than the homologous suspension or in which agglutination was questionable were studied further by means of the test tube agglutination technic.

The final dilutions of serums in the test tube method were between 1:20 and 1:5,120. The mixtures were incubated for two hours in a water bath at 37 C, then stored in the refrigerator overnight. Readings were made the next day without the aid of a magnifying glass against a dark background and with indirect lighting. Controls were checked prior to reading and if the cells did not become evenly resuspended the test was discarded. Results were recorded as four plus for complete, three plus as incomplete and two plus as partial agglutination. The homologous titer of each serum was arbitrarily set as the highest dilution exhibiting three plus or stronger agglutination.

Absorption technic.—Absorbing antigens were prepared in some instances by drying, at 37 C, organisms which had been packed by centrifugation after successive washes of absolute alcohol and then of absolute ether. Otherwise a 0.5 cc amount of sedimented bacterial cells was used directly. This amount of antigen, packed by centrifugation, or its equivalent of dried cells was routinely mixed with 10 cc of undiluted serum and the mixture incubated at 37 C for two hours while in a water bath provided with a mechanical shaking device. No difference could be detected between lots of serums absorbed with antigens prepared by the two methods.

RESULTS

The data from agglutination tests presented in table 1, indicate the interrelationships between nine strains of oral lactobacilli. Cross reactions which were considered to indicate an antigenic relationship are shown in boldface figures. One group relationship exists between strains #3, #16, #19 and #96, while another distinct group is comprised of strains #4, #9, #29 and #30. The antigens #3, #16 and #19 show cross reactions of higher titer with #45 antiserum than does antigen #45 with the antisera of strains #3, #16 and

#19. The antigenic relationship between strain #45 and the other three is apparently different from the relationships within that group, but the reactions were considered as showing a definite association between the four strains. On the other hand, strain #96, which is closely related antigenically to strains #3, #16 and #19, appears to have little or no antigenic material in common with

Agglutinin absorption tests

The results of selected agglutinin absorption tests are shown in table 2. There were insufficient amounts of antisera in the second group (see table 1) for complete titration after absorption hence two antisera, #29 and #30 were utilized. All the antisera were absorbed with the homologous organisms as well as with the heterologous ones

TABLE 1.—*Agglutination relationships of nine selected strains of oral lactobacilli.**

Anti-serums	Culture suspensions								%
	3	16	19	96	4	9	29	30	
3	5,120 2,560	5,120 1,280	1,280 1,280	5,120 1,280	20 —	40 40	— —	40 —	320 160
16	2,560 2,560	5,120 2,560	2,560 2,560	2,560 2,560	40 160	80 80	40 160	20 160	320 160
19	5,120 5,120	1,280 1,280	1,280 1,280	640 640	80 80	80 40	160 20	160 80	320 160
96	5,120 1,280	5,120 1,280	1,280 1,280	5,120 1,280	80 80	80 160	40 80	20 80	40 —
4	— —	— 80	— —	— —	5,120 1,280	640 640	5,120 1,280	5,120 1,280	80 80
9	20 40	— —	— —	80 —	640 640	1,280 1,280	1,280 1,280	1,280 1,280	80 20
29	— —	— 80	— —	— —	5,120 1,280	5,120 1,280	5,120 2,560	5,120 1,280	80 40
30	— —	— —	— —	— —	1,280 1,280	2,560 1,280	5,120 1,280	5,120 2,560	80 20
45	5,120 5,120	1,280 1,280	2,560 2,560	80 40	320 160	320 40	320 160	320 80	2,560 2,560

* Figures represent reciprocals of highest serum dilutions in which at least three plus agglutination occurred. Double entries represent reactions of antisera from duplicate rabbits. All control tests were negative. Normal serum reactions from the same animals prior to immunization are discussed in the text.

strain #45. Strain #96, therefore, is also somewhat different antigenically from strains #3, #16 and #19.

The light-faced figures denote reactions considered to be less significant, although some are as high as some of the titers for strain #45 which have been considered significant, because strains #4, #9, #29 and #30 were agglutinated in titers varying from 1:20 through 1:160 in nearly all sera from the nonimmunized animals and these are titers which were nearly as high as shown in immune sera. The significance of these so-called "normal" agglutinins will be discussed later.

shown in the table, but the results of these tests are not shown since absorption was complete in each instance and all agglutination tests performed with the homologously absorbed sera were negative in the lowest dilutions tested, 1:20.

It may be seen by comparison of the data shown in tables 1 and 2 that the antigenic differences indicated by agglutination tests with unabsorbed sera are more clearly apparent in the reactions of absorbed sera. For example, lactobacillus strain #30, which is a representative of the second agglutinative group (table 1), removed

TABLE 2.—*Agglutinin absorption tests.**

Anti-serums	Absorbed with antigen†	Culture suspensions tested									Antibodies in absorbed serums
		3	16	19	96	4	9	29	30	45	
3	Unabsorbed	5,120	5,120	1,280	5,120	—	40	—	—	320	A, B A A B
	30	1,280	1,280	640	160	—	—	—	—	320	
	45	1,280	640	640	320	—	—	—	—	—	
	96	1,280	1,280	640	—	—	—	—	—	320	
96	Unabsorbed	5,120	5,120	1,280	5,120	80	160	40	80	40	A
	29	1,280	1,280	640	640	—	—	—	—	—	
29	Unabsorbed	—	80	—	—	5,120	5,120	5,120	5,120	80	C C C
	45	—	—	—	—	1,280	640	2,560	1,280	—	
	96	—	—	—	—	2,560	1,280	2,560	2,560	—	
30	Unabsorbed	—	—	—	—	1,280	640	1,280	2,560	20	C
	3	—	—	—	—	640	640	640	1,280	—	
45	Unabsorbed	5,120	1,280	2,560	160	320	320	320	320	2,560	D, (Z) B, D B, D D D
	3	—	—	—	—	160	80	160	160	2,560	
	96	1,280	1,280	1,280	—	—	—	—	—	2,560	
	29	2,560	1,280	2,560	—	—	—	—	—	2,560	
	3, 96	—	—	—	—	—	—	—	—	2,560	

* Figures represent reciprocals of highest dilution in which three plus agglutination occurred.

† Each antiserum absorbed also with homologous organism. Completely negative agglutination reactions obtained with these absorbed serums.

from a first group antiserum (#3) all agglutinins for the organisms in the second group and markedly reduced the agglutinin content for strain #96, which had been found in agglutination tests with antiserum #45 to be different from the other members of the first agglutinative group; but such absorption only moderately reduced the titers originally present for organisms #3, #16 and #19, *Lactobacillus* strain #30 failed to remove the agglutinins for strain #45 from antiserum #3. Antigenic components, identified by the results of agglutination tests with the absorbed serums are seen in table 2.

Component A.—It has been noted previously that the low titered agglutination (table 1) of strains of the second group, in antisera of the first group, were due to antibodies present in the sera of the rabbits before immunization. There is no indication from either agglutination or agglutinin absorption tests that strain #3 possesses an antigenic component in common with strains #4, #9, #29 or #30. It is evident, however, that strains #3, #16 and #19 possess at least two antigenic components, of which only one is present in

strain #96. Absorption of antiserum #3 with #45 organisms removed agglutinins for #45 but the results were otherwise essentially the same as after absorption with #30. The designation "A" has been assigned to the antigenic component which is present in all four of these strains (#3, #16, #19 and #96).

Component B.—The previously noted difference between strain #96 and the other strains in the first agglutinative group is further clarified by the reactions of #3 antiserum after absorption with #96. Although in the original agglutination tests the reactions of #96 within the first group were essentially the same as the reactions of the others within that group, this strain absorbed agglutinins for itself from antiserum #3 without significant reduction of the titer of this serum either for strain #45 or for other organisms in the first agglutinative group. The designation "B" is assigned to the component absent from #96 but present in the other three (#3, #16 and #19). Neither A nor B is present in the strains of the second agglutinative group, #4, #9, #29 and #30.

Lactobacillus strain #45 does not possess antigenic component A since

it did not agglutinate in antiserum #96 to higher titer than it did in normal serum from the same animal prior to immunization and since its antiserum failed to cause agglutination of #96 organisms in higher titer than the normal serum agglutination of the same antigenic suspension. This strain (#45) does possess an antigenic component in common with strains #3, #16 and #19, however, and this may be assumed to be component B since there is not any evidence of an additional component common to these four strains. Antiserum #3, which contains A and B, is unaffected by absorption with lactobacillus strain #30, which contains neither A nor B, and it still agglutinated strain #45 after absorption with #30. When this antiserum was absorbed with strain #96, which does not contain B, it still agglutinated strain #45. The agglutination reactions (table 1) with strains #3, #16 and #19 in antiserum #45 occurred in as high titers as the agglutination of these strains in the homologous antisera but the reciprocal reactions, i.e., agglutination of #45 organisms in antisera #3, #16 and #19 were at much lower titers. The persistence of this titer after absorption of antiserum #3 with organisms not containing component B and its removal after absorption with strain #45 may be taken as additional evidence of its specificity. The fact that strain #45 is agglutinated to much higher titer in its homologous antiserum is, apparently, evidence that some antigenic component or components not common to strains #3, #16 and #19 combines more effectively with antibody than does its B antigenic component which is held in common with these strains.

Component C.—Agglutination tests have revealed that the antigenic components A and B were absent from the strains in the second agglutinative

group. The absence of these components was further shown by failure of strains containing either or both of the components A and B to absorb agglutinins from antisera #29 and #30. These results indicated the presence of a third component, "C," common to strains #4, #9, #29 and #30. Reciprocal absorptions within this group of strains (#4, #9, #29, #30) removed all agglutinins indicating that if any of these strains contain any component other than C such component or components are common to all of them.

Component D.—Lactobacillus strain #45, as indicated above, was found by agglutination and agglutinin absorption tests to contain antigen B and to lack antigens A and C. When antiserum #45 was absorbed with strain #3 organisms, the B antibody was removed so that strains #3, #16 and #19 were not agglutinated. However, this absorbed antiserum agglutinated #45 organisms to the full original titer of the antiserum as did the same antiserum after absorption with antigens A and C. This demonstrates the existence of a fourth antigenic component which is designated "D." A monoreactive serum for component D was obtained by successive absorptions of #45 antiserum with strains #3 and #96, the necessity for absorption with the latter strain (#96) is explained below.

Component (Z).—The possible presence of three components in the #45 antiserum was indicated when absorption of this serum with strain #3 organisms, i.e., antigenic components A and B, left "residual" agglutinins for organisms of the second agglutinative group (strains #4, #9, #29 and #30 which thus far had been shown to contain only component C) as well as for the homologous antigen #45. It is believed that these second group antibodies were present in the serum prior to im-

munization of the animal with #45 organisms. Evidence for this may be found in the fact that the preimmunization serum sample agglutinated #4, #9, #29 and #30 organisms to approximately the same titers. As it will appear from subsequent data, it seems unlikely that these residual or preimmunization antibodies were the same as antibody C since absorption of #45 antiserum with #96 organisms removed them and other tests indicate that strain #96 does not contain any other component in common with these strains. The designation "(Z)" is used for these residual antibodies. If (Z) is an antigenic component of the oral lactobacilli, it is present in strains #96 and #45 as well as in the strains containing the C component.

The presence of (Z) in #45 antisera, as well as in others with which strains #4, #9, #29 and #30 reacted, seems to be accounted for by persisting antibodies which were apparently present in the "normal" sera of the rabbits. It is noted in table 1 that antigens #45 and #96 demonstrated varying reactivity in the sera of rabbits immunized with strains #4, #9, #29 and #30 and it has been pointed out that these reactions are paralleled by similar reactivity in the sera of the same rabbits prior to parenteral injections. In order to obtain further information concerning these observations a series of absorption tests were performed with some of the normal sera. It was found that absorption with strain #4 which was frequently reactive in these sera removed all of the agglutinins. Strain #96 and #45 which also reacted occasionally in these sera also removed the (Z) component. That these results are not to be interpreted on the basis of non-specific absorption is evidenced by the fact that antigens #3 and #16, which along with strain #19 were rarely reac-

tive, did not remove agglutinins for strains #4, #9, #29 and #30 when used to absorb sera containing the (Z) component. The results seem to indicate, therefore, that component (Z) is contained in strains #4, #9, #29, #30, #45 and #96, but is not one of the major antigens in these strains. The nature of the (Z) component, if it is an entity, was such that no monoreactive (Z) serum could be obtained by the methods used.

Antigenic formulae.—The agglutinin absorption tests have shown the existence of four major antigenic components and a less definite, although a seemingly specific, fifth component among the cultures used in this study. On the basis of the reactivity in monospecific sera it is possible to write antigenic formulae for the nine strains. Some of the formulae in table 3, such as those for strains #16, #19, #96 and #29, were verified by the use of absorbed, specific, monoreactive sera.

It was of interest to determine the distribution of the four antigenic components among thirty other stock lactobacillus strains by testing them in the monoreactive sera. The reactivity in rapid-slide or test tube agglutination tests observed with eighteen of the strains is shown in table 4. The remaining twelve strains did not react in any of these sera by either slide or test

TABLE 3.—*Antigenic formulae of selected strains of oral lactobacilli.*

Strains	Morphological group (Hadley)	Monospecific antisera*			
		A (3/45)	B (3/96)	C (29/45)	D (45/3, 96)
3	I	+	+	—	—
16	I	+	+	—	—
19	III	+	+	—	—
96	I	+	—	—	—
4	II	—	—	+	—
9	III	—	—	+	—
29	I	—	—	+	—
30	I	—	—	+	—
45	II & III	—	+	—	+

* Parenthetical designation: numerator indicates antiserum and denominator absorbing organism, e.g., "A" antibody is obtained by absorbing #3 antiserum with #45 organisms.

TABLE 4.—*Source, morphology and presumptive antigenic formulae of stock cultures of lactobacilli reacting in monospecific serums.*

Strain numbers	Source	Morphological group (Hadley)	Components			
			A	B	C	D
26	Saliva	Intermediate	+	—	—	—
47	Saliva	II	+	—	—	—
107	Saliva	?	+	—	—	—
111AC	Saliva	III	+	—	—	—
116	Saliva	?	+	—	—	—
RH5G	Saliva	II	+	—	—	—
RH37E	Saliva	II	+	—	—	—
EH41N	Plaque	I	+	—	—	—
LB6	Vagina	II	+	—	—	—
49	Saliva	Intermediate	+	+	—	—
RH74B	Saliva	I	+	+	—	—
LB7	Vagina	II	+	+	—	—
EH41D	Plaque	II	—	—	+	—
RH24C	Saliva	II	—	—	+	—
RH43E	Saliva	II?	—	—	+	—
18	Saliva	III	+	+	+	—
32	Saliva	I	+	+	+	+
Zille	Intestine	I	+	+	+	+

Determinations are based on the results of slide agglutination tests. Questionable results were checked by test tube agglutination.
All control tests, bacterial suspensions in equivalent saline dilution, were negative.

tube agglutination. It is evident, from the fact that twelve cultures were negative in all tests, that all of the antibody components are not represented in the four monospecific serums used. It is noted that over half of the positive agglutinations occurred in the serum containing component A, but reactions occurred also in the other serums. No correlation could be found between these results and the morphological groups established by Hadley and Bunting (1930). Detailed study of the strains was not attempted and further study may reveal the presence of other antigenic components.

DISCUSSION

Agglutination tests with oral lactobacilli have been traditionally difficult to interpret because of irregularity in the reactions and because of spontaneous agglutination. It has been thought possible that one of the causes for irregularity in agglutination may be due to the use of long periods of immunization and/or large amounts of culture vaccine.

In a previous study (Williams and

Harrison, 1942) low agglutinin titers were found in serums from rabbits made hypersensitive to carbohydrate extracts of oral lactobacilli by repeated intradermal injections of heat-killed suspensions of the whole cells. Well defined agglutinative relationships among certain of the cultures became evident after heterologous tests were completed with the serums. Higher titered serums, which were prepared by the usual immunization procedure of repeated intravenous inoculation of small amounts of heat-killed oral lactobacilli, revealed that the relationships were characteristic of these serums. The data obtained indicated that this method of immunization yielded serums which could be used to begin a study of the main components in selected cultures of oral lactobacilli.

Since extended immunization is likely to show an inaccurate reflection of the bacteria inoculated, because antigen in lesser proportion may provoke an undue amount of antibody (Arkwright, 1931; Hooker and Boyd, 1934), rabbits were immunized over as short an interval and with as small an amount of heat-killed culture suspension as possible. In addition, each culture vaccine was used in more than one animal and the serums tested separately in order to control varying reactions in different rabbits (Beckwith, 1931; Hooker, 1937).

In many cases, spontaneous agglutination has been overcome by special treatment of the cultures, the cell suspensions or both. Suspension in broth (Howitt, 1930), phosphate buffered physiological sodium chloride (Canby and Bernier, 1942); suspension in distilled water (Weiss and Rettger, 1934); trituration of clumps (Hilgers, 1924); rapid and repeated transfer of cultures (Hunt and Rettger, 1930) have been some of the methods used. It was found in this study that stable suspen-

sions could be prepared from the majority of the cultures by washing the cells several times in large volumes of distilled water after centrifugation from broth. The use of distilled water for washing the cells was effective in suppressing clumping, but some of the cultures required dispersion by shaking the distilled water suspension with glass beads, filtering or both. Suspensions prepared by these means were stable and could be stored and re-used over intervals of more than one month.

The antigenic analysis was performed in a manner similar to the procedure suggested by Burrows and associates (1946) for cholera vibrios. Reciprocal absorptions demonstrated that four antigenic components, designated A, B, C and D, could be identified and monospecific serums prepared.

In preliminary tests rabbit antisera for cultures #3, #96, #30 and #45, which had been absorbed by the same methods as used in the antigenic analysis for separation of components, were checked for reactivity in precipitation tests with carbohydrate extracts from these four cultures. The tests showed the presence of the same components, in these absorbed serums, as had been found by the agglutination tests and seem to indicate that the carbohydrate precipitating antibody and the agglutinating antibody are identical. No evidence was obtained in these tests to suggest whether the reactivity of the (Z) component is due to a contaminating protein or to carbohydrates. These relationships are being explored in further studies.

When thirty other stock cultures were tested in the monospecific serums it was found that eighteen were agglutinated by one or more of the four types of serums. It should be noted that a few of these stock cultures had been isolated from intestinal and vaginal

tracts of human beings (table 4) and that they possessed immunological as well as morphological and cultural characteristics in common with the cultures from saliva and dental plaques. It seems probable from these tests that lactobacilli from these sources all belong to the *Lactobacillus acidophilus* species. The four antigenic components identified in the analysis represent only part of the antigens present in lactobacilli since twelve out of thirty cultures did not react in any of the serums. No correlation could be established between the morphological characteristics of these strains and the combinations of antigenic components.

The presence of a poorly defined component, designated (Z), was indicated in some of the antisera from rabbits immunized with the nine original cultures, but it was never found except in low titer and was masked by other components in high titer so that no monoreactive serum could be produced. This (Z) component has been found in the serums of several rabbits which had not been immunized parenterally and it appears likely, from the data thus far obtained, that it is a naturally existing or naturally acquired antibody. The short-term immunization procedure used in these studies may have excluded or delayed the development of antibodies to (Z) in some animals even when the component was present, if antibodies to it develop slowly, so that the main reactive components would have been emphasized and the (Z) component not detected. It is possible that serums prepared over a longer immunization period accompanied by utilization of larger total amounts of vaccine may have yielded additional information.

Strains which because of cross agglutination reactions were suspected of containing (Z) were found to absorb the reactive agglutinin from the normal

serums. Although many strains apparently do contain the (Z) component it is not universally present because strains of the first agglutinative group (#3, #16, #19) did not absorb it from the serums either before or after immunization. Reported findings of agglutinins in human serums prior to immunization may have been due to this type of antigenic component. Individuals whose titers before immunization were high invariably developed higher titers for the strains used in the vaccines than those individuals with low or without detectable titer (Williams, 1944). The broad reactivity of the (Z) component seems to indicate that the titers for lactobacilli in serums from "normal" human beings may be due to the presence of (Z) in the strains used in the tests. It appears, therefore, that before significance can be attached to agglutinative titers for oral lactobacilli in the serums of individuals with or without dental caries (Jay, Crowley, Hadley and Bunting, 1933) further studies are needed to determine the types or groups of cultures most closely associated with the carious process as well as the specific antigenic components, including (Z), which may be so associated.

In these investigations it has been demonstrated that definite groups of oral lactobacilli may be differentiated by immunological methods and it has been possible to clarify some of the previously uncertain immunological relationships. Successful use of the macroscopic slide agglutination test in these studies indicates that the technic should be very useful in screening strains for more detailed and more extended study. The results of the antigenic analysis promise to be particularly useful in the study of the relationships of these microorganisms to dental caries. Further study of the oral strains, particularly those from

dental plaques, is necessary to determine other antigenic components so that methods may be established for characterizing various cultures according to their antigenic structure.

SUMMARY

Nine lactobacillus cultures of human oral origin were used to prepare anti-serums in rabbits by repeated intravenous injection of small amounts of washed, heat-killed suspensions of the organisms.

An antigenic analysis of the cultures by agglutination and agglutinin absorption tests led to the identification and preparation of monospecific serums for four distinct antigenic components designated A, B, C, and D.

Evidence was found for the presence of one poorly defined antigenic component called (Z) and its significance is discussed.

Presumptive antigenic formulae for eighteen out of thirty other stock cultures of lactobacilli were obtained from either rapid-slide or test tube agglutination tests in the four monospecific serums.

The possibility for useful application of the results of antigenic analysis to the study of the etiological relationships of oral lactobacilli to dental caries is suggested.

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